Immunomodulatory effects of lovastatin on ovalbumin-induced bronchial asthma in mice

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ABSTRACT

Objectives: Lovastatin (LOV) is a cholesterol-lowering agent with immunomodulatory and anti-inflammatory effects. The present study evaluated the immunomodulatory effects of LOV in a mouse model of bronchial asthma. Methods: Mice were sensitized by giving 50 μg ovalbumin (OVA) i.p. with 1 mg alum on days 0 and 12. From day 22, mice were exposed to OVA (1% (w/v) in saline for 30 min, three times every 4th day. Negative control received saline similarly. Oral LOV, given 31 days, was starting from day 0 to day 30 and at sensitization day; it was given 30 min before the treatment. The number of inflammatory cells, levels of interleukin (IL)-4, IL-5, IL-13 and interferon (IFN)-γ in bronchoalveolar lavage fluid (BALF), serum IgE, OVA-specific IgE, IgG1 and IgG2a levels, in addition to histopathological and immunohistochemical examination of the lung were investigated. Results: LOV showed significant decrease in the number of leukocytes, macrophages and eosinophils, levels of IL-4, IL-5 and IL-13 in BALF, serum levels of IgE, OVA-specific IgE and IgG1, but no significant effect on BALF level of IFN-γ and serum level of OVA-specific IgG2a, in addition to the improvement of the histopathological and immunohistochemical changes. Conclusion: These results suggest that LOV could be beneficial for the treatment of bronchial asthma.

INTRODUCTION

Bronchial asthma is an inflammatory disease caused by a dysfunctional response of the immune system to normal, harmless environmental stimuli with accumulation of inflammatory cells, in particular, T lymphocytes, eosinophils, macrophages, mast cells and neutrophils in the bronchial airway mucosa [1, 2]. This was manifested by reversible recurrent episodes of wheezing chest, breathlessness, chest tightness and cough, especially at night and early in the morning in susceptible people [3]. Although, bronchial asthma is a multifactorial disease, Th2 cytokines, such as, interleukin (IL)-4, IL-5 and IL-13 are believed to play a crucial role in asthma development and progression. These cytokines are involved in overproduction of IgE, activation and increase of eosinophils in the airways and development of airway hyperresponsiveness (AHR) [4]. On the other hand, Th1 cytokines, among them interferon (IFN)-γ, were found to be able to suppress allergic airway inflammation [5]. The current strategies for the management of asthma aim to suppress airway inflammation, which is the key factor in asthma, however, airway remodeling is poorly responsive to such therapies through which the inhaled corticosteroid and beta-2 agonists are used, but with several side effects, especially when they are used in high doses or for a prolonged time [6, 7]. Moreover, they do not alter the underlying inflammatory process of the bronchial airways [8, 9]. So, the need for alternative safe and specific new anti-allergic drugs with fewer side effects is needed to palliate this allergic disorder. Allergic airway inflammation murine models are able to reproduce most of the salient features of human asthma. So, they are used for detecting the underlying mechanisms of airway inflammation and to validate the efficacy of newly obtained therapeutic agents for such allergy [10].

Lovastatin (LOV) is a member of statin class of the drugs that competitively inhibits 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme, used for lowering blood cholesterol level [11]. It has pleiotropic effects in preventing and treating the cardiovascular diseases with the prevention of strokes and thrombus formation, stabilization of atheromatous plaques, and improving endothelial functions [12-14]. Moreover, it has beneficial...
effects in treating multiple sclerosis [15], status epilepticus [16], brain inflammation [17], autoimmune uveoretinitis [18], glomerulonephritis [19] as well as graft-versus-host disease following bone marrow transplantation [20] by its immunomodulatory and anti-inflammatory effects. Recently, Chiba et al. [21] reported that LOV inhibits bronchial hyperresponsiveness through suppression of geranylgeranyltransferase-I upregulation. However, the immunomodulating and anti-inflammatory effects of LOV in allergic airway inflammation is still unclear.

The present study aimed to investigate the immunomodulatory as well as the anti-inflammatory effects of LOV in a mouse model of ovalbumin (OVA)-induced allergic asthma considering antigen induced inflammatory infiltration in the bronchial airways, cytokine levels in bronchoalveolar lavage fluid (BALF), immunoglobulin serum levels, serum cholesterol level, and histopathological as well as immunohistochemical changes of lung tissues.

MATERIALS AND METHODS

Reagents

Ovalbumin (Seikagaku Kogyo; Tokyo, Japan), lovastatin (Toronto Research Chemicals; Ontario, Canada), sodium carboxymethyl cellulose (Nacalai Tesque; Kyoto, Japan), calcium-magnesium-free phosphate buffer saline (PBS, Nissui Pharmaceutical Co; Tokyo, Japan), bovine serum albumin (BSA, Millipore; Kankakee, IL, USA), Block-Ace (Yukijirushi Co; Sapporo, Japan), Turk solution (Wako Pure Chemical Industries; Osaka, Japan), sodium pentobarbitone (Abbott Lab; Chicago, IL, USA), disodium ethylenediaminetetraacetic acid (EDTA-2Na) and acetylcarnoline chloride (Ach) (Nacalai Tesque, Japan), Diff-Quick solution (Sysmex; Kobe, Japan), pancuronium bromide (Sigma; St. Louis, MO, USA), monoclonal anti-dinitrophenol (DNP) IgE antibody (monoclonal anti-DNP IgE, clone SPE-7, Sigma), peroxidase-labeled polyclonal anti-mouse IgE goat IgG antibody (Nordic Immunological Laboratories; Tilburg, Netherlands), monoclonal rat anti-mouse IgE antibody (LO-ME-3, Serotec Co; Oxford, UK), polyclonal goat anti-mouse IgG1 antibody (STAR81; Serotec), monoclonal rat anti-mouse IgG2a antibody (LO-MG2a-7; Serotec), peroxidase-conjugated streptavidin (Dakopatts A/S; Glostrup, Denmark), and anti-E-cadherin antibody (Abcam, USA) were used.

Animals

Eight-week-old BALB/c mice were obtained from Japan SLC, Hamamatsu, Japan. They were housed in plastic cages in an air conditioned room at 22 ± 1°C with humidity of 60 ± 5%, fed a standard laboratory diet and given water ad libitum.

In this research 3 groups (8 mice each) were used; one group served as a control group through which mice were given saline by injection, the OVA-induced bronchial asthma group which received 0.5% sodium carboxymethyl cellulose (CMC) as vehicle, and the LOV-treated group in which the OVA-exposed animals were given LOV in a dose of 1 mg/kg/day (suspended in 0.5% CMC, prepared fresh daily).

The experiments were carried out following the guideline for the use and care of experimental animals in Gifu Pharmaceutical University with an approval of the University Animal Experiment Committee.

Sensitization and challenge

In accordance to Nagai et al. [22], mice were sensitized by 50 μg OVA with 1 mg alum intraperitoneally (i.p.) on days 0 and 12 and from day 22, mice were exposed to OVA (1% (w/v) in sterile physiological saline for 30 min, three times every 4th day. For negative control, mice were exposed to saline similarly. The generation of the aerosol (particle size 2-6 μm) done using a nebulizer (Ultrasonic Nebulizer UN-701, Azwell Co; Osaka, Japan) and was driven by filling a perspex cylinder chamber (diameter 5.5 cm, height 12 cm) with a nebulized solution. As regards LOV, it was administered orally for 31 days starting from day 0 to day 30 and was given 30 min before the treatment on the day of sensitization and challenge. After the final antigen challenge by 24 hours, BALF was collected and lung specimens obtained for histopathology and immunohistochemistry [22].

Bronchoalveolar fluid study

The inflammatory cell accumulation in BALF was examined according to Tanaka et al. [23]. In brief, after the final inhalation of antigen (day 31) by 24 hours, the animals were killed by injecting sodium pentobarbitone i.p. at a dose of 100 mg/kg. The left bronchus was tied and the right air lumen was washed four times using 0.5 ml of PBS containing 0.1% BSA and 0.05 mM EDTA-2Na. This was repeated three times with a total volume of 1.5 ml and recovery > 85%. BALF from each animal was pooled in a plastic tube, cooled in ice, then centrifuged (80g) at 4°C for 10 min with the cell pellets resuspended in the same buffer (1 ml). Mixing of the cell suspension with a Turk solution through which counting of the nucleated cells was done in a Burker chamber. By using a smear prepared with a cytospin centrifuge (Cytospin II, Shandon Ltd; Cheshire, UK), differential cell count was made through staining with Diff-Quick. Counting of 300 cells was done at a magnification of 500.

Measurement of cytokine levels in BALF

The BALF supernatant was used for measuring the amount of cytokines by enzyme-linked immunosorbent assay (ELISA) with a detection limit of each kit as 5 pg/ml for IL-4, 10 pg/ml for IFN-γ, 7 pg/ml for IL-5 and 1.5 pg/ml for IL-13, respectively. Kits for IL-4 and IFN-γ were obtained from Pierce Biotechnology (Rockford, IL, USA) and kits for IL-5 and IL-13 from R&D Systems (Minneapolis, MN, USA).
Measurement of immunoglobulin (Ig) levels in serum

Blood collection was done at day 30 immediately after the last inhalation and then by centrifugation sera were obtained and stored at -30°C. The levels of total serum IgE, OVA-specific IgE, OVA-specific IgG1 and OVA-specific IgG2a were measured using ELISA as described by Nagai et al. [24]. Briefly, the total serum IgE measured through coating flat-bottomed 96-well microtiter plates (Immunoplate I-96F, Nunc; Roskilde, Denmark) with monoclonal rat anti-mouse IgE antibody at a concentration of 5 μg/ml. Then after blocking with 1% Block-Ace, standard and serum dilutions were incubated for 1 h, followed by peroxidase-labeled polyclonal anti-mouse IgE goat IgG antibody. For standardization the diluted monoclonal anti-DNP IgE (SPE-7) was used.

Regarding serum OVA-specific IgE, serum OVA-specific IgG1 and serum OVA-specific IgG2a, they were measured by coating the microtiter plates with monoclonal rat anti-mouse IgE antibody (LO-ME-3) at a concentration of 5 μg/ml, polyclonal goat anti-mouse IgG1 antibody (STARS1) at a concentration of 2 μg/ml, and OVA solution at a concentration of 20 μg/ml, respectively. After blocking with 1% BSA, serum dilutions were incubated for 1 h, followed by biotinylated-OVA and peroxidase-conjugated streptavidin for serum OVA-specific IgE, serum OVA-specific IgG1 and by peroxidase-conjugated monoclonal rat anti-mouse IgG2a antibody (LO-MG2a7) for serum OVA-specific IgG2a. For standardization, diluted monoclonal anti-OVA IgE, IgG1, and IgG2a (donated by Dr. Kiniwa, Taiho Pharmaceutical Co; Tsukuba, Japan) was used.

By using an automatic ELISA plate reader (Multiscan MS ver 8; Lab systems Oy; Helsinki, Finland) at 492 nm (reference 690 nm), the optical densities were read and analyzed using Deltasoft 3 (Biometallics; Princeton, NJ, USA). The detection limit was 1 ng/ml for total IgE, anti-OVA IgE and anti-OVA IgG1, and was 5 ng/ml in case of anti-OVA IgG2a.

Measurement of serum cholesterol level

The serum cholesterol level was measured using ELISA kits (Bioassay Systems; Hayward, CA, USA). The detection limit of the kit was 1 mg/dl.

Histopathological study of lung specimens

Tissue specimens were obtained from the mid zone of the left lung of mice and immediately fixed in 10% formalin buffered saline; dehydrated in grades of alcohol and cleared in xylol. Impregnation was followed by embedding in hard paraffin. Sections of 5 μm obtained, dehydrated, cleared and mounted in Canada balsam. Finally staining with Haematoxylin & Eosin (H&E), Periodic acid Schiff’s (PAS) and Masson’s trichrome; then examined under light microscope [25].

Immunohistochemical localization of E-cadherin of bronchial epithelial cells

Immunohistochemical staining for E-cadherin was performed through placing the slides in 0.3% hydrogen peroxide/methanol for 20 min, followed by immersion in 10 ml of citrate buffer (pH) and autoclaved for 10 min, then left to cool at room temperature. Treating with a serum-free protein blocking solution for 20 min, then incubation overnight at 4°C with a 1:200 dilution of anti-E-cadherin antibody. Slides washed in PBS buffer, and incubated with polyvalent anti-mouse antibody for 10 min. 1-2 drop of DAB (3,3’-diaminobenzidine) applied to the sections for 5-10 min then counterstained with Mayer’s hematoxylin, dehydrated and cleared with xylol. At last sections examined and scored by light microscope for bronchial epithelial membrane, cytoplasmic and nuclear reaction [25].

Measurement of goblet cell number

In each slide of the different experimental groups, 3 to 5 randomly selected airways were photographed and circumferences of all airways were measured for goblet cell numbers. For standardization, goblet cell numbers in 100 μm were analyzed by division of total goblet cell number to the total length of airway circumferences and multiplying the result by one hundred.

Measurement of subepithelial collagen thicknesses

By using Leica Q500 MCO analyzer, collagen thickness was measured at x 400 through which 10 non-overlapping selected fields from each slide of the different experimental groups were examined.

Statistical analysis

The obtained values from the present research are presented as the mean ± standard error of the mean (SEM). The statistical significance was determined by using the two-tailed Student’s t-test or the Mann-Whitney’s U-test after evaluation by F-test. P ≤ 0.05 was considered to be significant.

RESULTS

Effect of LOV on antigen-induced airway inflammatory infiltrations

Repeated OVA inhalation in sensitized mice significantly increased the number of total leukocytes, macrophages and eosinophils in the BALF that was significantly inhibited by treatment with LOV (Figure 1).

Effect of LOV on BALF cytokine levels

In OVA-induced bronchial asthma group, the levels of IL-4, IL-5 and IL-13 in BALF, were significantly increased, while the level of BALF IFN-γ was significantly decreased. LOV treatment significantly suppressed the increased levels of IL-4, IL-5 and IL-13 in the BALF with no significant effect on the decreased level of BALF IFN-γ (Figure 2).
Effect of LOV on serum Igs

OVA-induced bronchial asthma significantly increased the serum levels of total IgE, OVA-specific IgE, IgG1 and IgG2a. In the LOV treated group, significantly inhibition of the increased levels of total IgE, OVA-specific IgE and IgG1 was found, while there was no significant effect on the increased level of OVA-specific IgG2a (Figure 3).

Effect of LOV on serum cholesterol level

Neither OVA sensitization and challenge nor LOV treatment showed significant difference in the serum level of cholesterol from saline sensitized and challenged group (Figure 4).

Histopathological results

H&E staining: Examination of the control group, revealed normal histological structure and architecture of the lung tissue with alveoli, bronchi, bronchioles and blood vessels. In OVA-induced bronchial asthma group, detached areas of the bronchial epithelial lining in association with goblet cell hyperplasia and mucus in the bronchial lumen were encountered. Perivascular and peribronchial mononuclear cellular infiltration as well as an apparent increase of the bronchial smooth muscle thickening and congested blood vessels was also seen. Oral administration of LOV markedly improves these histopathological findings (Figure 5, Table 1).
PAS staining: Normal goblet cell lining in the control group was observed. Marked goblet cell hyperplasia seen in the OVA-induced bronchial asthma group that was reversed to be nearly normal in LOV treated group (Figure 6). Regarding the statistical results of the number of goblet cells, there was a significant increase in their number in OVA group when compared to control, which, on the other hand, was found to be markedly lower in LOV treated group in comparison to OVA treated group (Figure 7).

Masson’s trichrome staining: Normal subepithelial collagen fibers in control was observed to be increased in OVA-induced bronchial asthma group. Considering LOV treated group normal subepithelial collagen content was observed (Figure 8). With regard to subepithelial collagen thickness there was a significant increase in collagen fibers in OVA group when compared to the control group and obviously lowered in LOV treated group in comparison to OVA group (Figure 9).

E-cadherin immunohistochemical results: In the control group, the bronchial epithelial cells showed positive

<table>
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<tr>
<th>Histopathological findings</th>
<th>Control</th>
<th>Ovalbumin</th>
<th>Lovastatin</th>
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<tbody>
<tr>
<td>Peribronchial cellular infiltration</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Bronchial muscle thickening</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Goblet cell hyperplasia</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Detachment of the bronchial epithelial lining</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Congested blood vessels</td>
<td>0</td>
<td>2</td>
<td>0</td>
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0, no findings; 1, mild affection; 2, moderate affection; 3, sever affection
DISCUSSION

The present research investigated the immunomodulatory effects of oral LOV in an allergic asthma mouse model in accordance to airways’ antigen-induced inflammatory infiltrations, local cytokines, levels of serum Ig and cholesterol, histopathological and immunohistochemical changes of lung tissues. The main results showed that oral treatment with LOV in mice sensitized and challenged with OVA significantly decreases the number of total leukocytes, macrophages and eosinophils, levels of IL-4, IL-5 and IL-13 in BALF, serum levels of total IgE, OVA-specific IgE and IgG1 with amelioration of histopathological and immunohistochemical changes in the lung. However, it showed no effect on the increased levels of BALF IFN-γ and serum OVA-specific IgG2a.
The Th2-type cytokines (IL-4, IL-5 and IL-13) play very important roles in the development of allergic airways inflammation and hyperresponsiveness in humans as well as in animal models [28, 29] through which IL-4 is essential for the differentiation and proliferation of T cells as well as it stimulates B cells to produce IgG1 and IgE [30-32]. IL-5 is an important cytokine for the differentiation, and maturation of eosinophils with subsequent increased levels in the bronchial airways [33, 34]. IL-13 plays an important role in the development of airways allergic manifestations with the increased production of goblet cell mucous [29, 35]. On the other hand Th1 cytokine (IFN-γ) enhance the production of IgG2a and suppresses allergic asthma manifestations [36, 37].

LOV has been shown previously to have immunomodulatory and anti-inflammatory effects in many animal models including, multiple sclerosis [15], status epilepticus [16], brain injury, inflammation [17], autoimmune uveoretinitis [18], glomerulonephritis [19] and graft-versus-host disease following bone marrow transplantation [20]; however, such kind of effects have never been shown in allergic airways inflammations. In the present study, oral LOV treatment suppressed IL-4, IL-5 and IL-13 production; but, it had no effect on the decreased level of IFN-γ in the BALF of antigen sensitized and challenge mice. These indicated that LOV treatment acts mainly by suppressing Th2-type and not Th1-type T cells. Moreover, the modulation of local Th1/Th2 cytokine production could explain the reduction of airway inflammation and cellular infiltration, and reduction of the level of total IgE, OVA-specific IgE and IgG1 as well as the failure of affecting the increased level of IgG2a, as our previous results show [27, 38]. In addition, it is important to clarify that LOV could have such immunomodulatory and anti-inflammatory effects when administered orally, as, this is the clinical administration route of the drug.

Furthermore, Michalik et al [39] have shown that LOV attenuates fibroblast to myofibroblast transition in bronchial airways of asthmatic patients by reducing the intracellular cholesterol level. The reduction of intracellular...

Table 2. Scoring of E-cadherin immunoreactivity in the different groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ovalbumin</th>
<th>Lovastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic reaction</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Membranous Reaction</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Nuclear Reaction sels</td>
<td>-</td>
<td>+</td>
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(+) positive immune reaction; (-) negative immune reaction

The selection of our model in inducing allergic airway inflammation occurs as it shows the different features of allergic airway inflammation in humans, including the production of different immunoglobulins, cytokines as well as histopathological findings [26, 27]. Thus, it is an ideal model to investigate the immunomodulatory effect of LOV on the allergic airway inflammation.
The different members of statins have been recently known to have distinguishable biochemical functions and may have not the same therapeutic potential [40]. Using a murine model of allergic asthma, Zeki et al. [41] have demonstrated that i.p. simvastatin is able to reduce airway inflammatory infiltration and BALF IL-4, IL-13 and tumor necrotizing factor (TNF)-α. Moreover, Imamura et al. [42] have shown that treatment with i.p. pravastatin suppressed eosinophic airway inflammation, serum OVA specific IgE and thoracic lymph node IL17. In addition, Samson et al. [43] have reported that treatment with fluvastatin to cultured peripheral blood mononuclear cells, obtained from patients with allergic asthma, resulted in suppression of its proliferation and reduction of IL-5 production. Interestingly, these data are matched with our finding, using oral LOV treatment.

In contrast, Chiba et al. [44, 45] have stated that i.p. LOV treatment did not inhibit the release of inflammatory Th2 cytokines (IL-4 and IL-13) in BALF and serum IgE production (total and OVA-specific IgE), even though it reduced inflammatory cell infiltration, especially eosinophils. This controversy may be due to different vehicle used (50% dimethyl sulfoxide), different method of sensitization and challenge, the amount of OVA used in sensitization, different route of administration and short time of treatment. Moreover, Chiba et al. [44], in rat model of asthma, have reported that i.p. LOV suppressed antigen induced airways hyperresponsiveness by inhibiting a monomeric GTP-binding protein (RhoA)-mediated signaling and not through suppression of Th2 cytokines (IL4, IL6 and IL13) and serum IgE level. This contradictory may be also due to different animal used, different vehicle used (50% dimethyl sulfoxide), different method of sensitization and challenge, different route of administration and short time of treatment.

Although, LOV is a member of statin class of the drugs which used for lowering blood cholesterol level [11], neither OVA sensitized and challenged nor LOV treated groups showed significant difference in the serum level of cholesterol in comparison to control group. This is most probably due to short duration of treatment which may be unable to suppress cholesterol synthesis; furthermore, the mice strain used in the present study is normal BALB/c and received standard laboratory diet which didn’t induced hyperlipidemia.

In the present study, detached epithelial lining was observed by immunohistochemical localization using E-cadherin antibody in OVA-sensitized animals. The decreased expression of bronchial epithelial cells was recorded by Xiao et al. [47] who showed that E-cadherin expression was significantly lower in the bronchial biopsies of asthmatic subjects compared with non-asthmatics ones. Song et al. [48] demonstrated that the activation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) is involved in the epithelial cells with subsequent alterations of E-cadherin distribution in bronchial epithelium. Kulshreshtha et al. [49] added that E-cadherin is a transmembrane protein in epithelial cells and provides essential architectural structure and immunological function to the airway epithelium.

From the mentioned data, we can conclude that, LOV had immunomodulatory effects in OVA-induced asthma in mice due to inhibition of Th2 cytokine production. So, LOV can be recommended as a therapeutic adjunctive for treatment of allergic bronchial asthma.
REFERENCES


Balaha et al: Lovastatin immunomodulation of bronchial asthma